FINAL REPORT

Test Facility Study No. 517562

Evaluation of the Mutagenic Activity of MLA-3202 in an in vitro Mammalian Cell Gene Mutation Test with L5178Y Mouse Lymphoma Cells

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COMPLIANCE STATEMENT

The study was performed in accordance with the OECD Principles of Good Laboratory Practice as accepted by Regulatory Authorities throughout the European Union, United States of America, Japan, and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

Exceptions from the above regulations are listed below.

- Concentration, stability, and homogeneity of test item formulations were not determined in this study. However, to limit the impact, the test item preparation was performed with approved procedures and documented in detail. Preparations were visually inspected for homogeneity prior to use and all preparations were used within 4 hours after preparation of the formulation.

This study was conducted in accordance with the procedures described herein. All deviations authorized/acknowledged by the Study Director are documented in the Study Records. The report represents an accurate and complete record of the results obtained.

There were no deviations from the above regulations that affected the overall integrity of the study or the interpretation of the study results and conclusions.

Date: 08 August 2017

C.M. Verspeek-Rip

Study Director

QUALITY ASSURANCE STATEMENT

Study title: Evaluation of the mutagenic activity of MLA-3202 in an in vitro mammalian cell gene mutation test with L5178Y mouse lymphoma cells.

This report was inspected by the Test Facility Quality Assurance Unit (QAU) according to the Standard Operating Procedure(s). The reported method and procedures were found to describe those used and the report reflects the raw data. The Test Facility inspection program was conducted in accordance with Standard Operating Procedure. During the on-site process inspections, procedures applicable to this type of study were inspected.

The dates of Quality Assurance inspections are given below.

Test Facility Study No. 517562

Type of Inspections	**Phase/Process**	**Start Inspection date**	**End Inspection date**	**Reporting date to TFM and SD***
Study	Study Plan	11-Apr-2017	11-Apr-2017	11-Apr-2017
	Study Plan Amendment 01	26-Jul-2017	26-Jul-2017	26-Jul-2017
	Report	02-Aug-2017	03-Aug-2017	03-Aug-2017
Process	**Test Item Receipt**	08-May-2017	15-May-2017	16-May-2017
	Test Item Handling			
	Genetic and In Vitro Toxicology	15-May-2017	31-May-2017	31-May-2017
	Exposure			
	Observations/Measurements			
	Specimen Handling			
	Test Item Handling			

*TFM=Test Facility Management SD = Study Director
The review of the final report was completed on the date of signing this QA statement.

Ulrich Wiets
Quality Assurance Auditor

U Wiets Date: 08 August 2017

1. RESPONSIBLE PERSONNEL

1.1. Test Facility

Study Director	C.M. Verspeek-Rip
Test Facility Management	E.J. van de Waart, MSc., ERT Head of Discovery & Environmental Sciences

1.2. Sponsor

Sponsor Representative / Study Monitor	Audrey Batoon, Ph.D.

2. SUMMARY

The objective of this study was to evaluate the mutagenic potential of MLA-3202 by testing its ability to induce forward mutations at the thymidine kinase (TK) locus in L5178Y mouse lymphoma cells, either in the absence or presence of a metabolic system (S9-mix). The TK mutational system detects base pair mutations, frame shift mutations and small deletions.

The test was performed in the absence of S9-mix with 3 and 24 hour treatment periods and in the presence of S9-mix with a 3 hour treatment period.

The study procedures described in this report were based on the most recent OECD guideline.

Batch RC-1045 of MLA-3202 was a clear amber-red liquid. The test item was dissolved in dimethyl sulfoxide.

In the first experiment, MLA-3202 was tested up to concentrations of 35 and 90 µg/ml in the absence and presence of S9-mix, respectively. The incubation time was 3 hours. The relative total growth (RTG) was 1 and 16% in the absence and presence of S9-mix, respectively. MLA-3202 precipitated in the culture medium at the dose level of 90 µg/ml.

In the second experiment, MLA-3202 was tested up to concentrations of 22.5 µg/ml in the absence of S9-mix. The incubation time was 24 hours. The RTG was 15%. MLA-3202 did not precipitate in the culture medium at this dose level.

The mutation frequency found in the solvent control cultures was within the range of the acceptability criteria of this assay and within the 95% control limits of the distribution of the historical concurrent solvent control database, except in the first experiment in the absence of S9-mix in which the mutation frequency of the solvent control cultures was not within the range of the acceptability criteria. Since the mutation frequency was just below the lower limit of the acceptability criteria range and and clear negative results are observed in all experiments, this deviation in the mutation frequency had no effect on the validity of the results of the second mutation experiment.

Positive control chemicals, methyl methanesulfonate and cyclophosphamide, both produced significant increases in the mutation frequency. In addition, the mutation frequency found in the positive control cultures was within the 95% control limits of the distribution of the historical positive control database. It was therefore concluded that the test conditions were adequate and that the metabolic activation system (S9-mix) functioned properly.

In the absence of S9-mix, MLA-3202 did not induce a significant increase in the mutation frequency in the first experiment. This result was confirmed in an independent experiment with modification in the duration of treatment.

In the presence of S9-mix, MLA-3202 did not induce a significant increase in the mutation frequency.

In conclusion, MLA-3202 is not mutagenic in the mouse lymphoma L5178Y test system under the experimental conditions described in this report.

3. INTRODUCTION

The objective of this study was to evaluate the mutagenic potential of MLA-3202 by testing its ability to induce forward mutations at the thymidine kinase (TK) locus in L5178Y mouse lymphoma cells, either in the absence or presence of a metabolic system (S9-mix). The TK mutational system detects base pair mutations, frame shift mutations and small deletions.

Background of the test system

L5178Y mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes. The TK mutational system is able to detect base pair alterations, frame shift mutations and small deletions and clastogenic effect.

Cells deficient in thymidine kinase (TK), due to the forward mutation ($TK^{+/-}$ to $TK^{-/-}$) are resistant to the cytotoxic effects of the pyrimidine analogue trifluorothymidine (TFT).

TK deficient cells cannot incorporate the analogue into its phosphorylated derivative (nucleotide); the nucleotides needed for cellular metabolism are obtained solely from *de novo* synthesis. In the presence of TK, TFT is converted into nucleotides, which is lethal to the cells. Thus, cells that are able to proliferate in culture medium containing TFT are mutated, either spontaneously or by the action of the test item, to a TK deficient phenotype. Furthermore, by applying the TFT-selection procedure it is possible to discriminate between two different classes of TFT-resistant mutants (small and large colonies). The large colonies are believed to be the result of mutants with single gene mutations (substitutions, deletions of base-pairs) affecting the TK gene. The small colonies are believed to be the result of chromosomal damage to the TK and adjacent genes.

A test article, which induces a positive response in this assay, is presumed to be a potential mammalian cell mutagen.

The design of this study is based on the following study guideline:

- OECD Guideline 490. *"Genetic Toxicology: In Vitro Mammalian Cell Gene Mutation Test Using the Thymidine Kinase Gene"*, (adopted 29 July 2016).

The Study Director signed the study plan on 12 Apr 2017. The experimental start date was 18 Apr 2017, and the experimental completion date was 03 Jul 2017.

The study plan and deviations are presented in Appendix 1.

4. MATERIALS AND METHODS

4.1. Test Item, Vehicle and Reference Items

4.1.1. Test Item

Test item information

Identification	MLA-3202
Appearance	Clear amber-red liquid
Batch	RC-1045
Purity/Composition	UVCB
Test item storage	At room temperature
Stable under storage conditions until	17 February 2019 (expiry date)

Additional information

Test facility test item number	207258/A
Purity/composition correction factor	No correction factor required
Test item handling	No specific handling conditions required
Stability at higher temperatures	Stable
Chemical name (IUPAC), synonym or trade name	Amides, tallow, N,N-bis(2-hydroxypropyl)
CAS Number	1454803-04-3
Solubility in vehicle	Dimethyl sulfoxide: Not indicated
Stability in vehicle	Dimethyl sulfoxide: Not indicated

4.2. Vehicle

The vehicle for the test item was dimethyl sulfoxide (Merck Darmstadt, Germany).

4.3. Reference Items

4.3.1. Negative Control

The negative control was dimethyl sulfoxide, the vehicle of the test item.

4.3.2. Positive Controls

4.3.2.1. Without Metabolic Activation

Methyl methanesulfonate (MMS); CAS No. 66-27-3 (purity 98%, Sigma Aldrich GmbH, Steinheim, Germany). MMS was used as a direct acting mutagen at concentrations of 15 and 5 µg/ml for the 3 and 24 hour treatment periods, respectively. MMS was dissolved in dimethyl sulfoxide. The stock solutions of MMS were prepared immediately before use.

4.3.2.2. With Metabolic Activation

Cyclophosphamide (CP); CAS No. 50-18-0 (purity 100%, Baxter B.V., Utrecht, The Netherlands). CP was used as an indirect acting mutagen, requiring metabolic activation, at a final concentration of 7.5 µg/ml. CP was dissolved in Hanks' balanced salt solution (HBSS) (Life Technologies, Bleiswijk, The Netherlands) without calcium and magnesium. The stock solutions of CP were stored in aliquots at ≤-15°C in the dark and one sample was thawed immediately before use.

4.4. Test Item Characterization

The Sponsor provided to the Test Facility documentation of the identity, purity, composition, and stability for the test item(s). A Certificate of Analysis or equivalent document was provided to the Test Facility and is presented in Appendix 2.

4.5. Reserve Samples

For each batch (lot) of test item, a reserve sample (about 0.5 gram) was collected and maintained under the appropriate storage conditions by the Test Facility and destroyed after the expiration date.

4.6. Test Item Inventory and Disposition

Records of the receipt, distribution, and storage of test item(s) were maintained. With the exception of reserve samples, all unused Sponsor-supplied test item will be discarded or returned to the Sponsor after completion of the scheduled program of work. Records of the decisions made will be kept at the Test Facility.

4.7. Dose Formulation and Analysis

4.7.1. Preparation of Test Item

No correction was made for the purity/composition of the test item.

A solubility test was performed based on visual assessment. The test item was dissolved in dimethyl sulfoxide.

Test item concentrations were used within 2 hours of preparation.

The final concentration of the solvent in the exposure medium was 1.0% (v/v).

Any residual volumes were discarded.

4.8. Test System

Test System	L5178Y/TK$^{+/-}$-3.7.2C mouse lymphoma cells.
Rationale	Recommended test system in international guidelines (e.g. OECD).
Source	American Type Culture Collection, (ATCC, Manassas, USA) (2001).

Stock cultures of the cells were stored in liquid nitrogen (-196°C). The cultures were checked for mycoplasma contamination. Cell density was kept below 1 x 10^6 cells/ml.

4.8.1. Cell Culture

Horse serum

Horse serum (Life Technologies) was inactivated by incubation at 56°C for at least 30 minutes.

Basic medium

RPMI 1640 Hepes buffered medium (Dutch modification) (Life Technologies) containing penicillin/streptomycin (50 U/ml and 50 µg/ml, respectively) (Life Technologies), 1 mM sodium pyruvate (Sigma, Zwijndrecht, The Netherlands) and 2 mM L-glutamin (Life Technologies).

Growth medium

Basic medium, supplemented with 10% (v/v) heat-inactivated horse serum (=R10 medium).

Exposure medium

For 3 hour exposure:

Cells were exposed to the test item in basic medium supplemented with 5% (v/v) heat-inactivated horse serum (R5-medium).

For 24 hour exposure:

Cells were exposed to the test item in basic medium supplemented with 10% (v/v) heat-inactivated horse serum (R10-medium).

Selective medium

Selective medium consisted of basic medium supplemented with 20% (v/v) heat-inactivated horse serum (total amount of serum = 20%, R20-medium) and 5 µg/ml trifluorothymidine (TFT) (Sigma).

Non-selective medium

Non-selective medium consisted of basic medium supplemented with 20% (v/v) heat-inactivated horse serum (total amount of serum = 20%, R20-medium).

Environmental conditions

All incubations were carried out in a humid atmosphere (80 - 100%, actual range 64 - 98%) containing 5.0 ± 0.5% CO_2 in air in the dark at 37.0 ± 1.0°C (actual range 35.0 - 37.7°C). Temperature and humidity were continuously monitored throughout the experiment. The CO_2 percentage was monitored once on each working day. Temporary deviations from the temperature, humidity and CO_2 percentage may occur due to opening and closing of the incubator door. Any variation to these conditions were evaluated and maintained in the raw data.

4.8.2. Metabolic Activation System

Rat liver microsomal enzymes (S9 homogenate) were obtained from Trinova Biochem GmbH, Giessen, Germany and was prepared from male Sprague Dawley rats that have been dosed orally with a suspension of phenobarbital (80 mg/kg body weight) and ß-naphthoflavone (100 mg/kg body weight).

4.8.2.1. Preparation of S9-Mix

S9-mix was prepared immediately before use and kept on ice. S9-mix components contained per ml physiological saline: 1.63 mg $MgCl_2.6H_2O$ (Merck); 2.46 mg KCl (Merck); 1.7 mg glucose-6-phosphate (Roche, Mannheim, Germany); 3.4 mg NADP (Randox Laboratories Ltd., Crumlin, United Kingdom); 4 µmol HEPES (Life technologies). The above solution was filter (0.22 µm)-sterilized. To 0.5 ml S9-mix components 0.5 ml S9-fraction was added (50% (v/v) S9-fraction) to complete the S9-mix.

The concentration of the S9-fraction in the exposure medium was 4% (v/v).

4.9. Experimental Design

4.9.1. Cleansing

Prior to dose-range finding and mutagenicity testing, the mouse lymphoma cells were grown for 1 day in R10-medium containing 10^{-4} M hypoxanthine (Sigma), 2 x 10^{-7} M aminopterine (Fluka Chemie AG, Buchs, Switzerland) and 1.6 x 10^{-5} M thymidine (Sigma) (HAT-medium) to reduce the amount of spontaneous mutants, followed by a recovery period of 2 days on

R10-medium containing hypoxanthine and thymidine only. After this period cells were returned to R10-medium for at least 1 day before starting the experiment.

4.9.2. Dose-range Finding Test

In order to select appropriate dose levels for mutagenicity testing, cytotoxicity data were obtained by treating 4 x 10^6 cells (10^6 cells/ml for 3 hour treatment) or 6 x 10^6 cells (1.25 x 10^5 cells/ml for 24 hour treatment) with a number of test item concentrations increasing by approximately half log steps (see study plan deviation, Appendix 1). The cell cultures for the 3 hour treatment were placed in sterile 30 ml centrifuge tubes, and incubated in a shaking incubator at 37.0 ± 1.0°C and 145 rpm. The cell cultures for the 24 hour treatment were placed in sterile 75 cm^2 culture flasks at 37.0 ± 1.0°C. The test item was tested in the absence and presence of S9-mix.

Since the test item was poorly soluble in the exposure medium, the highest tested concentration was 156 µg/ml exposure medium.

For the 3 hour treatment, cell cultures were exposed to the test item in exposure medium in the absence as well as in the presence of S9-mix. After exposure, the cells were separated from the treatment solutions by 2 centrifugation steps (216 g, 5 min). The first centrifugation step was followed by removal of the supernatant and resuspension of the cells in Hanks' balanced salt solution and after the second centrifugation step the cells were resuspended in 50 ml growth medium (R10-medium).

For the 24 hour treatment, cell cultures were exposed to the test item in exposure medium in the absence of S9-mix. After exposure, the cells were separated from the treatment solutions by 2 centrifugation steps (216 g, 5 min). The first centrifugation step was followed by removal of the supernatant and resuspension of the cells in Hanks' balanced salt solution and after the second centrifugation step the cells were resuspended in 20 ml growth medium (R10-medium). The cells in the final suspension were counted with the coulter particle counter.

The surviving cells of the 3 hour treatment were subcultured twice to determine cytotoxicity. After 24 hour of subculturing, the cells were counted and subcultured again for another 24 hours, after that the cells were counted. The surviving cells of the 24 hour treatment were subcultured once. After 24 hours of subculturing, the cells were counted. If less than 1.25 x 10^5 cells/ml were counted no subculture was performed.

The suspension growth expressed as the reduction in cell growth after approximately 24 and 48 hours or only 24 hours cell growth, compared to the cell growth of the solvent control, was used to determine an appropriate dose-range for the mutagenicity tests.

4.9.3. Mutagenicity Test

Eight doses of the test item were tested in the absence and presence of S9-mix with a 3 hour treatment period and six dose levels were tested in the absence of S9-mix with a 24 hour treatment period.

The highest doses that were tested gave a cell survival of approximately 10-20% and the survival in the lowest doses was approximately the same as the cell survival in the solvent control (see study plan deviation, Appendix 1). Also some intermediate doses were tested.

4.9.4. Treatment of the Cells

Per culture 8 x 10^6 cells (10^6 cells/ml for 3 hour treatment) or 6 x 10^6 cells (1.25 x 10^5 cells/ml for 24 hour treatment) were used. The cell cultures for the 3 hour treatment were placed in sterile 30 ml centrifuge tubes, and incubated in a shaking incubator at 37.0 ± 1.0°C and 145 rpm. The cell cultures for the 24 hour treatment were placed in sterile 75 cm^2 culture flasks at 37.0 ± 1.0°C. Solvent and positive controls were included and the solvent control was tested in duplicate.

In the first experiment, cell cultures were exposed for 3 hours to MLA-3202 in exposure medium in the absence and presence of S9-mix. In the second experiment, cell cultures were exposed to MLA-3202 in exposure medium for 24 hours in the absence of S9-mix.

For the 3 hour treatment, cell cultures were exposed to the test item in exposure medium in the absence as well as in the presence of S9-mix. After exposure, the cells were separated from the treatment solutions by 2 centrifugation steps (216 *g*, 5 min). The first centrifugation step was followed by removal of the supernatant and resuspension of the cells in Hanks' balanced salt solution and after the second centrifugation step the cells were resuspended in 50 ml growth medium (R10-medium).

For the 24 hour treatment, cell cultures were exposed to the test item in exposure medium in the absence of S9-mix. After exposure, the cells were separated from the treatment solutions by 2 centrifugation steps (216 g, 5 min). The first centrifugation step was followed by removal of the supernatant and resuspension of the cells in Hanks' balanced salt solution and after the second centrifugation step the cells were resuspended in 20 ml growth medium (R10-medium). The cells in the final suspension were counted with the coulter particle counter.

4.9.5. Expression Period

For expression of the mutant phenotype, the remaining cells were cultured for 2 days after the treatment period. During this culture period at least 4 x 10^6 cells (where possible) were subcultured every day in order to maintain log phase growth. Two days after the end of the treatment with the test item the cells were plated for determination of the cloning efficiency (CE_{day2}) and the mutation frequency (MF).

4.9.6. Determination of the Mutation Frequency

For determination of the CE_{day2} the cell suspensions were diluted and seeded in wells of a 96-well dish. One cell was added per well (2 x 96-well microtiter plates/concentration) in non-selective medium.

For determination of the mutation frequency (MF) a total number of 9.6 x 10^5 cells per concentration were plated in five 96-well microtiter plates, each well containing 2000 cells in selective medium (TFT-selection), with the exception of the positive control groups (MMS and CP) where a total number of 9.6 x 10^5 cells/concentration were plated in ten 96-well microtiter plates, each well containing 1000 cells in selective medium (TFT-selection). The microtiter plates for CE_{day2} and MF were incubated for 11 or 12 days. After the incubation period, the plates for the TFT-selection were stained for 2 hours, by adding 0.5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma) to each well. The plates for the CE_{day2} and MF were scored with the naked eye or with the microscope.

4.9.7. Analysis of Results

4.9.7.1. Determination of the Mutant Colonies

The colonies were divided into small and large colonies. Mutant cells that have suffered extensive genetic damage have prolonged doubling times and thus form small colonies. Less severely affected mutant cells grow at rates similar to the parental cells and form large colonies. The small colonies can be associated with the induction of chromosomal mutations. The large colonies appear to result from mutants with single gene mutations (substitutions, deletions of base-pairs) affecting the TK gene.

The small colonies are morphologically dense colonies with a sharp contour and with a diameter less than a quarter of a well. The large colonies are morphologically less dense colonies with a hazy contour and with a diameter larger than a quarter of a well. A well containing more than one small colony is classified as one small colony. A well containing more than one large colony is classified as one large colony. A well containing one small and one large colony is classified as one large colony.

4.9.7.2. Calculation of the Survival or Viability

Dose-range finding test:

The suspension growth (SG) for the 3 hour treatment=
SG = Suspension growth = [Day 1 cell count/1.6 x 10^5] x [Day 2 cell count/1.25 x 10^5]

The suspension growth (SG) for the 24 hour treatment=
SG = Suspension growth = [Day 0 cell count/1.25 x 10^5] x [Day 1 cell count/1.25 x 10^5]

Mutagenicity tests:

The suspension growth (SG) for the 3 hour treatment=
[Day 1 cell count/1.6 x 10^5] x [Day 2 cell count/1.25 x 10^5]

The suspension growth (SG) for the 24 hour treatment=
[Day 0 cell count/1.25 x 10^5] x [Day 1 cell count/1.25 x 10^5] x [Day 2 cell count/1.25 x 10^5]

Relative Suspension Growth (RSG) = $SG_{(test)} / SG_{(controls)} \times 100$

The cloning efficiency was determined by dividing the number of empty wells by the total number of wells. The value obtained is the P(0), the zero term of the Poisson distribution:
P(0) = number of empty wells/total number of wells

The cloning efficiency (CE) was then calculated as follows:
CE = -ln P(0)/number of cells plated per well

The relative cloning efficiency (RCE) at the time of mutant selection =
$CE_{(test)} / CE_{(controls)} \times 100$

The Relative Total Growth (RTG) was also be calculated as the product of the cumulative relative suspension growth (RSG) and the relative survival for each culture:
RTG = RSG x RCE/100

4.9.7.3. Calculation of the Mutation Frequency

The mutation frequency was expressed as the number of mutants per 10^6 viable cells. The plating efficiencies of both mutant and viable cells (CE_{day2}) in the same culture were determined and the mutation frequency (MF) was calculated as follows:

$MF = \{-\ln P(0)/\text{number of cells plated per well}\}/ CE_{day2} \times 10^6$

Small and large colony mutation frequencies were calculated in an identical manner.

5. ACCEPTABILITY CRITERIA

A mutation assay was considered acceptable if it met the following criteria:

a) The absolute cloning efficiency of the solvent controls (CE_{day2}) is between 65 and 120% in order to have an acceptable number of surviving cells analyzed for expression of the TK mutation.

b) The spontaneous mutation frequency in the solvent control is ≥ 50 per 10^6 survivors and ≤ 170 per 10^6 survivors.

c) The suspension growth (SG) over the 2-day expression period for the solvent controls should be between 8 and 32 for the 3 hour treatment, and between 32 and 180 for the 24 hour treatment.

d) The positive control should demonstrate an absolute increase in the total mutation frequency, that is, an increase above the spontaneous background MF (an induced MF (IMF)) of at least 300×10^{-6}. At least 40% of the IMF should be reflected in the small colony MF. And/or, the positive control has an increase in the small colony MF of at least 150×10^{-6} above that seen in the concurrent solvent control (a small colony IMF of 150×10^{-6}).

All results presented in the tables of the report are calculated using values as per the raw data rounding procedure and may not be exactly reproduced from the individual data presented.

6. ANALYSIS

In addition to the criteria stated below, any increase of the mutation frequency should be evaluated for its biological relevance including comparison of the results with the historical control data range.

The global evaluation factor (GEF) has been defined by the IWGT as the mean of the negative/solvent MF distribution plus one standard deviation. For the micro well version of the assay the GEF is 126.

A test item is considered positive (mutagenic) in the mutation assay if it induces a MF of more than $MF_{(controls)} + 126$ in a dose-dependent manner. An observed increase should be biologically relevant and will be compared with the historical control data range.

A test item is considered equivocal (questionable) in the mutation assay if no clear conclusion for positive or negative result can be made after an additional confirmation study.

A test item is considered negative (not mutagenic) in the mutation assay if: none of the tested concentrations reaches a mutation frequency of $MF_{(controls)} + 126$.

7. COMPUTERIZED SYSTEMS

Critical computerized systems used in the study are listed below. All computerized systems used in the conduct of this study have been validated; when a particular system has not satisfied all requirements, appropriate administrative and procedural controls were implemented to assure the quality and integrity of data.

Text Table 1
Critical Computerized Systems

System name	Version No.	Description of Data Collected and/or Analyzed
REES Centron	SQL 2.0	Temperature and humidity (laboratory facilities) Data collection

8. RETENTION OF RECORDS

All study-specific raw data, electronic data, documentation, study plan and final report(s) generated by Charles River from this study will be transferred to a Charles River archive no later than the date of final report issue unless otherwise specified in the study plan. At least five years after issue of the final report, the Sponsor will be contacted.

9. RESULTS

9.1. Solubility

MLA-3202 precipitated in the exposure medium at concentrations of 156 µg/ml and above, Therefore 156 µg/ml was used as the highest test item concentration.

9.2. Dose-range Finding Test

In the dose-range finding test, L5178Y mouse lymphoma cells were treated with a test item concentration range of 9.8 to 156 µg/ml in the absence of S9-mix with 3 and 24 hour treatment periods and in the presence of S9-mix with a 3 hour treatment period.

Table 1 shows the cell counts of the cultures after 3 hours of treatment with various concentrations of the test item and after 24 and 48 hours of subculture, the calculated suspension growth and the relative suspension growth.

In the absence of S9-mix, the relative suspension growth was 37% at the test item concentration of 39 µg/ml compared to the relative suspension growth of the solvent control. Hardly any or no cell survival was observed at test item concentrations of 78 µg/ml and above.

In the presence of S9-mix, the relative suspension growth was 79% at the test item concentration of 78 µg/ml compared to the relative suspension growth of the solvent control. Hardly any or no cell survival was observed at the test item concentration of 156 µg/ml.

Table 2 shows the cell counts of the cultures after 24 hours of treatment with various concentrations of the test item and after 24 hours of subculture and the calculated suspension growth and the relative suspension growth.

The relative suspension growth was 36% at the test item concentration of 20 µg/ml compared to the relative suspension growth of the solvent control. Hardly any or no cell survival was observed at test item concentrations of 39 µg/ml and above.

9.3. Mutation Experiment

Table 3 and Table 4 show the percentages of cell survival and the mutation frequencies for various concentrations of the test item. Individual colony counts of cloning and selective plates and cell counts during subculturing are listed in Table 5 to Table 11 of Appendix 4.

Initially a mutation experiment was performed with a 24 hour treatment period in the absence of S9-mix. However since due to a technical error, no acceptable responses of the spontaneous mutation frequency in the solvent controls were obtained, this experiment was rejected and no data will be reported.

9.3.1. First Mutagenicity Test

Based on the results of the dose-range finding test, the following dose-range was selected for the first mutagenicity test:
Without S9-mix: 5, 10, 20, 30, 35, 40, 50, 55, 60, 65 and 70 µg/ml exposure medium.
With S9-mix: 20, 40, 80, 90, 100, 110, 120, 130, 140 and 150 µg/ml exposure medium.

Evaluation of test item precipitation

The test item precipitated in the exposure medium at concentrations of 90 µg/ml and above.

Evaluation of toxicity

In the absence and presence of S9-mix, too many dose levels showed severe cytotoxicity, this experiment was repeated (experiment 1A): the following dose-ranges were selected:

Without S9-mix: 0.63, 1.25, 2.5, 5, 10, 20, 25, 30, 35, 40, 45 and 50 µg/ml exposure medium.
With S9-mix: 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/ml exposure medium.

In the absence of S9-mix, the dose levels of 0.63 to 20 µg/ml showed no cytotoxicity. Therefore, the dose level of 0.63 µg/ml was not regarded relevant for mutation frequency measurement. The dose levels of 40 µg/ml and above were not used for mutation frequency measurement, since these dose levels were too toxic for further testing.

In the presence of S9-mix, the dose levels of 5 to 60 µg/ml showed no cytotoxicity. Therefore, the dose levels of 10 and 40 µg/ml were not regarded relevant for mutation frequency measurement. The dose level of 100 µg/ml was not used for mutation frequency measurement, since this dose level was too toxic for further testing.

The dose levels selected to measure mutation frequencies at the TK-locus were:
Without S9-mix: 1.25, 2.5, 5, 10, 20, 25, 30 and 35 µg/ml exposure medium.
With S9-mix: 5, 20, 30, 50, 60, 70, 80 and 90 µg/ml exposure medium.

In the absence of S9-mix (Table 3), the relative total growth of the highest test item concentration was 1% compared to the total growth of the solvent controls, see study plan deviation, Appendix 1.

In the presence of S9-mix, the relative total growth of the highest test item concentration was 16% compared to the total growth of the solvent controls.

Evaluation of the mutagenicity

No significant increase in the mutation frequency at the TK locus was observed after treatment with MLA-3202 either in the absence or in the presence of S9-mix. The numbers of small and large colonies in the MLA-3202 treated cultures were comparable to the numbers of small and large colonies of the solvent controls.

9.3.2. Second Mutagenicity Test

To obtain more information about the possible mutagenicity of MLA-3202, a second mutation experiment was performed in the absence of S9-mix with a 24 hour treatment period.

Based on the results of the dose-range finding test and experiment 1, the following dose levels were selected for mutagenicity testing: 2.5, 5, 10, 15, 20, 22.5, 25, 27.5, 30 and 35 µg/ml exposure medium.

Evaluation of test item precipitation

The test item did not precipitate in the exposure medium up to and including the concentration of 35 µg/ml.

Evaluation of toxicity

The dose levels of 25 to 35 µg/ml were not used for mutation frequency measurement, since these dose levels were too toxic for further testing. The dose levels selected to measure mutation frequencies at the TK-locus were: 2.5, 5, 10, 15, 20 and 22.5µg/ml exposure medium.

The relative total growth of the highest test item was 15% compared to the total growth of the solvent controls, see Table 4.

Evaluation of mutagenicity

No significant increase in the mutation frequency at the TK locus was observed after treatment with MLA-3202. The numbers of small and large colonies in the MLA-3202 treated cultures were comparable to the numbers of small and large colonies of the solvent controls.

10. DISCUSSION

The mutation frequency found in the solvent control cultures was within the acceptability criteria of this assay and within the 95% control limits of the distribution of the historical negative control database. (See Appendix 5, Table 12). Except in the presence of S9-mix (first experiment), in which the mutation frequency of the solvent control cultures were just below the acceptability criteria of this assay (see study plan deviation, Appendix 1)

Positive control chemicals, methyl methanesulfonate and cyclophosphamide, both produced significant increases in the mutation frequency. In addition, the mutation frequency found in the positive control cultures was within the 95% control limits of the distribution of the historical positive control database. It was therefore concluded that the test conditions were adequate and that the metabolic activation system (S9-mix) functioned properly.

The suspension growth over the two-day expression period for cultures treated with DMSO was between 16 and 19 (3 hour treatment) and 94 and 95 (24 hour treatment) (See Appendix 4).

In the absence of S9-mix, MLA-3202 did not induce a significant increase in the mutation frequency in the first experiment. This result was confirmed in a repeat experiment with modification in the duration of treatment.

In the presence of S9-mix, MLA-3202 did not induce a significant increase in the mutation frequency.

11. CONCLUSION

In conclusion, MLA-3202 is not mutagenic in the TK mutation test system under the experimental conditions described in this report.

12. REFERENCES

1. D.E. Amacher, S.C. Paillet and V. Ray. Point mutations at the thymidine kinase locus in L5178Y mouse lymphoma cells. I. Application to genetic toxicology testing. Mutation Res., 64, 391-406 (1979).
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6. J. Cole and C.F. Arlett. The detection of gene mutations in cultured mammalian cells. Mutagenicity testing, a practical approach Ed. S. Venit, and J.M. Parry. IRL press limited (1984).
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9. EC Guideline No. 440/2008. *Part B: Methods for the Determination of Toxicity and other health effects, Guideline B.17: "Mutagenicity – In Vitro Mammalian Cell Gene Mutation Test". Official Journal of the European Union No. L142, 31 May 2008.*

Appendix 1
Study Plan and Deviations

STUDY PLAN

FINAL STUDY PLAN

Test Facility Study No. 517562

Evaluation of the Mutagenic Activity of MLA-3202 in an in vitro Mammalian Cell Gene Mutation Test with L5178Y Mouse Lymphoma Cells

SPONSOR:
Chemtura Corporation
199 Benson Road
MIDDLEBURY, CT 06749
USA

TEST FACILITY:
Charles River Laboratories Den Bosch BV
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

12 April 2017

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1. OBJECTIVE

The objective of this study is to evaluate the mutagenic potential of the test item by testing its ability to induce forward mutations at the thymidine kinase (TK) locus in L5178Y mouse lymphoma cells, either in the presence or absence of a metabolic system (S9-mix). The TK mutational system detects base pair mutations, frame shift mutations and small deletions.

Background of the test system

L5178Y mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes. The TK mutational system is able to detect base pair alterations, frame shift mutations and small deletions and clastogenic effect.

Cells deficient in thymidine kinase (TK), due to the forward mutation ($TK^{+/-}$ to $TK^{-/-}$) are resistant to the cytotoxic effects of the pyrimidine analogue trifluorothymidine (TFT).

TK deficient cells cannot incorporate the analogue into its phosphorylated derivative (nucleotide); the nucleotides needed for cellular metabolism are obtained solely from *de novo* synthesis. In the presence of TK, TFT is converted into nucleotides, which is lethal to the cells. Thus, cells that are able to proliferate in culture medium containing TFT are mutated, either spontaneously or by the action of the test item, to a TK deficient phenotype. Furthermore, by applying the TFT-selection procedure it is possible to discriminate between two different classes of TFT-resistant mutants (small and large colonies). The large colonies are believed to be the result of mutants with single gene mutations (substitutions, deletions of base-pairs) affecting the TK gene. The small colonies are believed to be the result of chromosomal damage to the TK and adjacent genes.

A test item, which induces a positive response in this assay, is presumed to be a potential mammalian cell mutagen.

2. PROPOSED STUDY SCHEDULE

Proposed study dates are listed below. Actual applicable dates will be included in the Final Report.

Experimental Start Date:	17 Apr 2017 (First date of study-specific data collection)
Experimental Completion Date:	02 Jul 2017 (Last date data are collected from the study)
Unaudited Draft Report:	16 Jul 2017

3. GUIDELINES FOR STUDY DESIGN

The design of this study was based on the study objective, the overall product development strategy for the test item and the following study design guidelines:

- OECD Guideline 490. *"Genetic Toxicology:* In Vitro *Mammalian Cell Gene Mutation Test Using the Thymidine Kinase Gene",* (adopted 29 July 2016).

4. REGULATORY COMPLIANCE

The study will be performed in accordance with the OECD Principles of Good Laboratory Practice as accepted by Regulatory Authorities throughout the European Union, United States of America, Japan, and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

5. QUALITY ASSURANCE

5.1. Test Facility

The Test Facility Quality Assurance Unit (QAU) will monitor the study to assure the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with Good Laboratory Practice regulations. The QAU will review the study plan, conduct inspections at intervals adequate to assure the integrity of the study, and audit the Final Report to assure that it accurately describes the methods and standard operating procedures and that the reported results accurately reflect the raw data of the study.

The Test Facility QAU contact is indicated below:

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6. SPONSOR

Sponsor Representative/ Sponsor Study Monitor

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8. TEST ITEM, VEHICLE AND REFERENCE ITEMS

8.1. Test Item

Test item information

Identification	MLA-3202
Appearance	Clear amber-red liquid
Batch	RC-1045
Purity/Composition	UVCB
Test item storage	At room temperature
Stable under storage conditions until	17 February 2019 (expiry date)

Additional information

Test facility test item number	207258/A
Purity/composition correction factor	No correction factor required
Test item handling	No specific handling conditions required
Stability at higher temperatures	Stable
Chemical name (IUPAC), synonym or trade name	Amides, tallow, N,N-bis(2-hydroxypropyl)
CAS Number	1454803-04-3

8.1. Vehicle

Solubility in vehicle:	
• Dimethyl sulfoxide	Not indicated
Stability in vehicle:	
• Dimethyl sulfoxide	Not indicated

8.2. Reference Items

8.2.1. Negative Control

The vehicle of the test item.

8.2.2. Positive Controls

8.2.2.1. Without Metabolic Activation

Methyl methane sulfonate (MMS); CAS No. 66-27-3. MMS will be used as a direct acting mutagen at a concentration of 15 µg/ml for a 3 hour treatment period and 5 µg/ml for a 24 hour treatment period. MMS will be dissolved in dimethyl sulfoxide. The stock solution of MMS will be prepared immediately before use.

8.2.2.2. With Metabolic Activation

Cyclophosphamide (CP); CAS No. 50-18-0. CP will be used as an indirect acting mutagen, requiring metabolic activation, at a final concentration of 7.5 µg/ml. CP will be dissolved in Hanks' balanced salt solution (HBSS) without calcium and magnesium. The stock solutions of CP are stored in aliquots in the freezer (≤ -15°C) in the dark and one sample will be thawed immediately before use.

8.3. Test Item Characterization

The Sponsor will provide to the Test Facility documentation of the identity, purity, composition, and stability for the test item(s) and vehicle(s). A Certificate of Analysis or equivalent documentation will be provided for inclusion in the Final Report. The Sponsor will also provide information concerning the regulatory standard that was followed for these evaluations.

The Sponsor has appropriate documentation on file concerning the method of synthesis, fabrication or derivation of the test item(s), and this information is available to the appropriate regulatory agencies should it be requested.

8.4. Analysis of Test Item

The stability of the bulk test item will not be determined during the course of this study. Information to support the stability of each lot of the bulk test item will be provided by the Sponsor.

8.5. Reserve Samples

For each batch (lot) of test item, if practically possible a reserve sample will be collected and maintained under the appropriate storage conditions by the Test Facility and destroyed after the expiration date.

8.6. Test Item Inventory and Disposition

Records of the receipt, distribution, storage, and disposition of test item(s) will be maintained.

9. SAFETY

The following safety instructions apply to this study:

Standard safety precautions specified in Charles River Den Bosch procedures.

10. DOSE FORMULATION AND ANALYSIS

10.1. Preparation of Test Item

No correction will be made for the purity/composition of the test compound.

The test items will be dissolved or suspended in dimethyl sulfoxide. Test item concentrations will be used within 4 hours after preparation. The final concentration of the solvent in the culture medium will not exceed 1% (v/v).

The pH and the osmolarity of the culture medium containing the highest tested concentration (if higher than or equal to 500 µg/ml and not precipitating) will be recorded.

Concentrations and vehicle will be approved by the study director in the study files.

Any residual volumes will be discarded unless otherwise requested by the Study Director.

10.2. Sample Collection and Analysis

Analysis of test item in vehicle for concentration, stability, homogeneity will not be performed, however, to limit the impact, the test item preparation will be performed with approved procedures and documented in detail. Formulations will be visually inspected for

homogeneity prior to use and all formulations will be used within 4 hours after adding vehicle to the test item. This GLP exception was therefore considered as being minor with no impact on the outcomes and the integrity and the achievement of the objective of the study.

11. TEST SYSTEM

Test System	L5178Y/TK$^{+/-}$-3.7.2C mouse lymphoma cells
Rationale	Recommended test system in international guidelines (e.g. OECD, EC).
Source	American Type Culture Collection, (ATCC, Manassas, USA) (2001)

Stock cultures of these cells are stored in liquid nitrogen (-196°C). The cultures are checked for mycoplasma contamination. Cell density will be preferably kept below 1 x 10^6 cells/ml.

11.1. Cell Culture

Horse serum

Horse serum will be inactivated by incubation at 56°C for at least 30 minutes.

Basic medium

RPMI 1640 Hepes buffered medium (Dutch modification) containing penicillin/streptomycin (50 U/ml and 50 µg/ml, respectively), 1 mM sodium pyruvate and 2mM L-glutamin.

Growth medium

Basic medium, supplemented with 10% (v/v) heat-inactivated horse serum (R10-medium).

Exposure medium

For 3 hour exposure:

Cells will be exposed to the test item in basic medium supplemented with 5% (v/v) heat-inactivated horse serum (R5-medium).

For 24 hour exposure:

Cells will be exposed to the test item in basic medium supplemented with 10% (v/v) heat-inactivated horse serum (R10-medium).

Selective medium

Selective medium consists of basic medium, supplemented with 20% (v/v) heat-inactivated horse serum (R20-medium) and 5 µg/ml trifluorothymidine (TFT).

Non-selective medium

Non-selective medium consists of basic medium, supplemented with 20% (v/v) heat-inactivated horse serum (R20-medium).

Environmental conditions

All incubations will be carried out in a humid atmosphere (80 - 100%) containing 5.0 ± 0.5% CO_2 in air in the dark at 37.0 ± 1.0°C. Temperature and humidity will be continuously monitored throughout the experiment. The CO_2 percentage will be monitored

once on each working day. Temporary deviations from the temperature, humidity and CO_2 percentage may occur due to opening and closing of the incubator door. Any variation to these conditions will be evaluated and maintained in the raw data.

11.2. Metabolic Activation System

Rat S9 homogenate will be obtained from Trinova Biochem GmbH, Giessen, Germany and is prepared from male Sprague Dawley rats that have been dosed orally with a suspension of phenobarbital (80 mg/kg body weight) and ß-naphthoflavone (100 mg/kg).

11.2.1. Preparation of S9-Mix

S9-mix will be prepared immediately before use and kept on ice. S9-mix components contains per ml physiological saline: 1.63 mg $MgCl_2.6H_2O$; 2.46 mg KCl; 1.7 mg glucose-6-phosphate; 3.4 mg NADP; 4 µmol HEPES. The above solution will be filter (0.22 µm)-sterilized. To 0.5 ml S9-mix components 0.5 ml S9-fraction will be added (50% (v/v) S9-fraction) to complete the S9-mix.

The concentration of the S9-fraction in the exposure medium will be 4% (v/v).

12. EXPERIMENTAL DESIGN

12.1. Cleansing

Prior to dose-range finding and mutagenicity testing, the mouse lymphoma cells will be grown for 1 day in growth medium (R10-medium) containing 10^{-4} M hypoxanthine, 2 x 10^{-7} M aminopterin and 1.6 x 10^{-5} M thymidine (HAT-medium) to reduce the amount of spontaneous mutants, followed by a recovery period of 2 days on medium containing hypoxanthine and thymidine only. After this period cells will be returned to R10-medium for at least 1 day before starting the experiment.

12.2. Dose-range Finding Test

In order to select appropriate dose levels for mutagenicity testing, cytotoxicity data will be obtained by treating 8 x 10^6 cells (10^6 c/ml for 3 hour treatment) or 6 x 10^6 cells (1.25 x 10^5 c/ml for 24 hour treatment), suspended in exposure medium with a range of test item concentrations increasing with approximately half log steps. The cell cultures for the 3hours treatment will be placed in sterile 30 ml centrifuge tubes, and incubated in a shaking incubator at 37.0 ± 1.0 °C and 145 rpm. The cell cultures for the 24 hours treatment will be placed in sterile 75 cm^2 culture flasks at 37.0 ± 1.0 °C. The test item will be tested in the absence and in the presence of S9-mix.

In case the test item will be difficult to dissolve in aqueous solutions, the highest dose level will be determined by the solubility in the culture medium. Concentrations exceeding 5 mg/ml (whichever is the lowest) will not be tested.

For the 3 hour treatment, cell cultures will be exposed for to the test item in exposure medium in the absence as well as in the presence of S9-mix. After exposure, the cells will be separated from the treatment solutions by 2 centrifugation steps (216 *g*, 5 min). The first centrifugation step will be followed by removal of the supernatant and resuspension of the cells in Hanks' balanced salt solution and after the second centrifugation step the cells will be resuspended in 50 ml growth medium (R10-medium).

For the 24 hour treatment, cell cultures will be exposed to the test item in exposure medium in the absence of S9-mix. After exposure, the cells will be separated from the treatment solutions by 2 centrifugation steps (216 *g*, 5 min). The first centrifugation step will be followed by removal of the supernatant and resuspension of the cells in Hanks' balanced salt solution and after the second centrifugation step the cells will be resuspended in 20 ml growth medium (R10-medium). The cells in the final suspension will be counted with the coulter particle counter.

The surviving cells of the 3 hours treatment will be subcultured twice to determine cytotoxicity. After 24 hours of subculturing, the cells will be counted and subcultured again for another 24 hours, after that the cells will be counted. The surviving cells of the 24 hours treatment will be subcultured once. After 24 hours of subculturing, the cells will be counted. If less than 1.25×10^5 cells/ ml are counted no subculture will be performed.

The suspension growth expressed as the reduction in cell growth after approximately 24 and 48 hours or only 24 hours cell growth, compared to the cell growth of the solvent control, will be used to determine an appropriate dose-range for the mutagenicity tests.

12.3. Mutagenicity Test

The test item will be tested in the presence of S9-mix with a 3 hour treatment period and in the absence of S9-mix with a 3 and 24 hour treatment period.

The highest doses that will be tested should give a cell survival of approximately 10-20% and the survival in the lowest doses should be approximately the same as the cell survival in the solvent control. Also some intermediate doses will be tested. In case the test item will not be toxic and/or difficult to dissolve in aqueous solutions, the highest concentration will be determined by the solubility in the culture medium. The highest test item concentrations may show a slight to heavy precipitate in the exposure medium. In general, concentrations exceeding 5 mg/ml will be not tested. Six to eight concentrations will be selected for the determination of the mutation frequency.

12.4. Treatment of the Cells

At least 8×10^6 cells (10^6 c/ml for 3 hour treatment) or 6×10^6 cells (1.25×10^5 c/ml for 24 hour treatment) will be used per culture. The cell cultures for the 3 hours treatment will be placed in sterile 30 ml centrifuge tubes, and incubated in a shaking incubator at 37.0 ± 1.0 °C and 145 rpm. The cell cultures for the 24 hour treatment will be placed in sterile 75 cm^2 culture flasks at 37.0 ± 1.0 °C. Solvent and positive controls will be included. The solvent control will be tested in duplicate.

For the 3 hour treatment, cell cultures will be exposed for to the test item in exposure medium in the absence as well as in the presence of S9-mix. After exposure, the cells will be separated from the treatment solutions by 2 centrifugation steps (216 *g*, 5 min). The first centrifugation step will be followed by removal of the supernatant and resuspension of the cells in Hanks' balanced salt solution and after the second centrifugation step the cells will be resuspended in 50 ml growth medium (R10-medium).

For the 24 hour treatment, cell cultures will be exposed to the test item in exposure medium in the absence of S9-mix. After exposure, the cells will be separated from the treatment solutions by 2 centrifugation steps (216 *g*, 5 min). The first centrifugation step will be

followed by removal of the supernatant and resuspension of the cells in Hanks' balanced salt solution and after the second centrifugation step the cells will be resuspended in 20 ml growth medium (R10-medium). The cells in the final suspension will be counted with the coulter particle counter.

12.5. Expression Period

For expression of the mutant phenotype, the remaining cells will be cultured for 2 days after the treatment period. During this culture period at least 4 x 10^6 cells (if possible) will be subcultured every day in order to maintain log phase growth. Two days after the end of the treatment with the test item, the cells will be plated for determination of the cloning efficiency (CE_{day2}) and the mutation frequency (MF).

12.6. Determination of the Mutation Frequency

For determination of the CE_{day2} the cell suspensions will be diluted and seeded in wells of a 96-well dish. One cell will be added per well (2 x 96-well microtiter plates/concentration) in non-selective medium.

For determination of the mutation frequency (MF) a total number of 9.6 x 10^5 cells/concentration will be plated in five 96-well microtiter plates, each well containing 2000 cells in selective medium (TFT-selection), with the exception of the positive control groups (MMS and CP) where a total number of 9.6 x 10^5 cells/concentration will be plated in ten 96-well microtiter plates, each well containing 1000 cells in selective medium (TFT-selection). The microtiter plates for CE_{day2} and MF will be incubated for 10-12 days.

After this incubation period the plates for the TFT-selection will be stained for 2 hours, by adding 0.5 mg/ml MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to each well. The plates for the CE_{day2} and MF will be scored with the naked eye or the microscope.

12.7. Analysis of Results

12.7.1. Determination of the Mutant Colonies

The colonies will be divided into small and large colonies. Mutant cells that suffer extensive genetic damage will have prolonged doubling times and thus form small colonies. Less severe affected mutant cells grow at rates similar to the parental cells and form large colonies. The small colonies can be associated with the induction of chromosomal aberrations. The large colonies appeared to result from mutants with single gene mutations (substitutions, deletions of base-pairs) affecting the TK gene.

The small colonies are morphological dense colonies with a sharp contour and with a diameter less than a quarter of a well. The large colonies are morphological less dense colonies with a hazy contour and with a diameter larger than a quarter of a well. A well containing more than one small colony is classified as one small colony. A well containing more than one large colony is classified as one large colony. A well containing one small and one large colony is classified as one large colony.

12.7.2. Calculation of the Survival or Viability

Dose-range finding test:

The suspension growth (SG) for the 3 hour treatment is:
SG = Suspension growth = [Day 1 cell count/1.6 x 10^5] x [Day 2 cell count/1.25 x 10^5]

The suspension growth (SG) for the 24 hour treatment is:
SG = Suspension growth = [Day 0 cell count/1.25 x 10^5] x [Day 1 cell count/1.25 x 10^5]

Mutagenicity tests:

The suspension growth (SG) for the 3 hour treatment is:

[Day 1 cell count/1.6 x 10^5 1)] x [Day 2 cell count/1.25 x 10^5 1)]

The suspension growth (SG) for the 24 hour treatment is:

[Day 0 cell count/1.25 x 10^5] x [Day 1 cell count/1.25 x 10^5 1)] x [Day 2 cell count/1.25 x 10^5 1)]

1) Or appropriate cell concentration

Relative Suspension Growth (RSG) = $SG_{(test)}$ / $SG_{(controls)}$ x 100

The cloning efficiency will be determined by dividing the number of empty wells by the total number of wells. The value obtained is the P(0), the zero term of the Poisson distribution:

P(0) = number of empty wells/total number of wells

The cloning efficiency (CE) is then calculated as follows:

CE = -ln P(0)/number of cells plated per well

The relative cloning efficiency (RCE) at the time of mutant selection =

$CE_{(test)}$ / $CE_{(controls)}$ x 100

The Relative Total Growth (RTG) will also be calculated as the product of the cumulative relative suspension growth (RSG) and the relative survival for each culture:

RTG = RSG x RCE/100

12.7.3. Calculation of the Mutation Frequency

The mutation frequency will be expressed as the number of mutants per 10^6 viable cells. The plating efficiencies of both mutant and viable cells (CE_{day2}) in the same culture will be determined and the mutation frequency (MF) will be calculated as follows:

MF = {-ln P(0)/number of cells plated per well}/ CE_{day2} x 10^6

Small and large colony mutation frequencies are calculated in an identical manner.

13. ACCEPTABILITY CRITERIA

A mutation assay is considered acceptable if it meets the following criteria:

a) The absolute cloning efficiency of the solvent controls (CE_{day2}) is between 65 and 120% in order to have an acceptable number of surviving cells analysed for expression of the TK mutation.

b) The spontaneous mutation frequency in the solvent control is ≥ 50 per 10^6 survivors and ≤ 170 per 10^6 survivors.

c) The suspension growth (SG) over the 2-day expression period for the solvent controls should be between 8 and 32 for the 3 hour treatment, and between 32 and 180 for the 24 hour treatment.

d) The positive control should demonstrate an absolute increase in the total mutation frequency, that is, an increase above the spontaneous background MF (an induced MF (IMF)) of at least 300 x 10^{-6}. At least 40% of the IMF should be reflected in the small colony MF. And/or, the positive control has an increase in the small colony MF of at least 150 x 10^{-6} above that seen in the concurrent solvent control (a small colony IMF of 150 x 10^{-6}).

If (one of) the acceptability criteria are not met and the Study Director decides that this has a critical effect on the study, the test will be rejected and repeated.

In case no clear conclusion for positive or negative result can be made an additional confirmation study will be performed to confirm the study results.

14. ANALYSIS

In addition to the criteria stated below, any increase of the mutation frequency should be evaluated for its biological relevance including a comparison of the results with the historical control data range.

The global evaluation factor (GEF) has been defined by the IWGT as the mean of the negative/solvent MF distribution plus one standard deviation. For the micro well version of the assay the GEF is 126 x 10^{-6} .

A test item is considered positive (mutagenic) in the mutation assay if it induces a MF of more than $MF_{(controls)}$ + 126 in a dose-dependent manner. A Cochran Armitage trend test ($p < 0.05$) will be performed to test whether there is a significant trend in the induction (ToxRat Professional v 3.2.1). Any observed increase should be biologically relevant and will be compared with the historical control data range.

A test item is considered equivocal (questionable) in the mutation assay if no clear conclusion for positive or negative result can be made after an additional confirmation study.

A test item is considered negative (not mutagenic) in the mutation assay if: none of the tested concentrations reaches a mutation frequency of $MF_{(controls)}$ + 126.

15. COMPUTERIZED SYSTEMS

The following critical computerized systems may be used in the study. The actual critical computerized systems used will be specified in the Final Report.

Data for parameters not required by study plan, which are automatically generated by analytical devices used will be retained on file but not reported. Statistical analysis results that are generated by the program but are not required by study plan and/or are not scientifically relevant will be retained on file but will not be included in the tabulations.

Critical Computerized Systems

System name	Description of Data Collected and/or Analyzed
REES Centron	Temperature and humidity (laboratory facilities) Data collection

16. AMENDMENTS AND DEVIATIONS

Changes to the approved study plan shall be made in the form of an amendment, which will be signed and dated by the Study Director. Every reasonable effort will be made to discuss any necessary study plan changes in advance with the Sponsor.

All study plan and SOP deviations will be documented in the study records. The Study Director will notify the Sponsor of deviations that may result in a significant impact on the study as soon as possible.

17. RETENTION OF RECORDS

All study-specific raw data, electronic data, documentation, study plan, retained samples and specimens (except perishable specimens) and final reports will be archived by no later than the date of final report issue. All materials generated by Charles River from this study will be transferred to a Charles River archive. At least five years after issue of the final report, the Sponsor will be contacted.

Records to be maintained will include, but will not be limited to, documentation and data for the following:

- Study plan, study plan amendments, and deviations
- Study schedule
- Study-related correspondence
- Test item receipt, identification and preparation
- Measurements and observations

18. REPORTING

A comprehensive Draft Report will be prepared following completion of the study and will be finalized following consultation with the Sponsor. The report will include all information necessary to provide a complete and accurate description of the experimental methods and results and any circumstances that may have affected the quality or integrity of the study.

The Sponsor will receive an electronic version of the Draft Report. The Final Report will be provided in Adobe Acrobat PDF format (hyperlinked and searchable) along with a Microsoft Word version of the text. The PDF document will be created from native electronic files to the extent possible, including text and tables generated by the Test Facility. Report components not available in native electronic files and/or original signature pages will be scanned and converted to PDF image files for incorporation. An original copy of the report with the Test Facility's handwritten signatures will be retained.

Reports should be finalized within 6 months of issue of the Draft Report. If the Sponsor has not provided comments to the report within 6 months of draft issue, the report will be finalized by the Test Facility unless other arrangements are made by the Sponsor.

19. REFERENCES

1. D.E. Amacher, S.C. Paillet and V. Ray. Point mutations at the thymidine kinase locus in L5178Y mouse lymphoma cells. I. Application to genetic toxicology testing. Mutation Res., 64, 391-406 (1979).
2. D.E. Amacher, S.C. Paillet, G.N. Turner, V.A. Ray and D.S. Salsburg. Point mutations at the thymidine kinase locus in L5178Y mouse lymphoma cells. II. Test validation and interpretation, Mutation Res., 72, 447-474 (1980).
3. B.N. Ames, J. Mc Cann and E. Yamasaki. Methods for detecting carcinogens and mutagens with the *Salmonella/mammalian* microsome mutagenicity test. Mutation Res., 31, 347-364 (1975).
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5. D. Clive and J.F.S. Spector. Laboratory procedures for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. Mutation Res., 31, 17-29 (1975).
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TEST FACILITY APPROVAL

The signature below acknowledges Test Facility Management's responsibility to the study as defined by the relevant GLP regulations.

Date: 12 April 2017

I.A.J. Verbaan, PhD.
Section Head Discovery

The signature below indicates that the Study Director approves the study plan.

Date: 12 April 2017

C.M. Verspeek-Rip
Study Director

SPONSOR APPROVAL

The signature of the Sponsor Representative below indicates approval of this study plan.

Audrey Batoon Date: 13 April 2017

Audrey Batoon, Ph.D.

Sponsor Representative

ATTACHMENT A

Distribution List

Electronic copies will be supplied unless otherwise specified below.

Version	Recipient
Original	Study Director
1 Copy	Sponsor Representative / Study Monitor
1 Copy	QAU / Management

charles river

STUDY PLAN AMENDMENT NO: 1

Study Title	Evaluation of the mutagenic activity of MLA-3202 in an *in vitro* mammalian cell gene mutation test with L5178Y mouse lymphoma cells
Sponsor	LANXESS Solutions US Inc. 199 Benson Road MIDDLEBURY, CT 06749 USA
Study Monitor	Audrey Batoon, Ph.D.
Test item	207258/A
Test Facility Study No.	517562

AMENDMENT DESCRIPTION

1. Front page, Sponsor's address, should read:

   LANXESS Solutions US Inc.
   199 Benson Road
   MIDDLEBURY, CT 06749
   USA

REASONS FOR AMENDMENT

1. The data has been changed according to information of the sponsor, dated 21 July 2017.

   This has no effect on other data.

APPROVAL
Study director

.....
C.M. Verspeek-Rip

24 July 2017
.....
date:

DEVIATIONS

All deviations that occurred during the study have been authorized/acknowledged by the Study Director, assessed for impact, and documented in the study records. All study plan deviations and those SOP deviations that could have impacted the quality or integrity of the study are listed below.

None of the deviations were considered to have impacted the overall integrity of the study or the interpretation of the study results and conclusions.

Dose range finding test

- In order to select appropriate dose levels for mutagenicity testing, cytotoxicity data were obtained by treating 4 x 10^6 cells (10^6 cells/ml for 3 hour treatment) or 6 x 10^6 cells (1.25 x 10^5 cells/ml for 24 hour treatment).
  Evaluation: The dose range finding test was only performed to select appropriate dose levels for mutagenicity testing, therefore the deviation in the amount of treated cells had no effect on the results of the study.

Mutagenicity test

- The survival of the highest tested dose levels (Relative total growth) in the first experiment (absence of S9-mix) were not within the range mentioned in the study plan (10-20%).
  Evaluation: Due to steep toxicity borders, the Relative total growth (RTG) of 35 µg/ml was 1%, whereas 30 µg/ml showed a RTG of 32%. However since, no increase in the mutation frequencies of more than two-fold were observed compared to the solvent controls; the testing of dose levels with a RTG of 10-20% would have given limited additional information.

Determination of the Mutant colonies

- In the first mutation experiment in the presence of S9-mix, the mutation frequency of the solvent control cultures was recorded to be outside the range of $\geq$ 50 per 10^6 survivors and $\leq$ 170 per 10^6 survivors.
  Evaluation: The values of 41 and 47 per 10^6 survivors were below the lower limit of the range (50 per 10^6 survivors). The mutation frequencies were just below the lower limit of the range and negative results are observed in all experiments, therefore this deviation in the mutation frequency had no effect on the results of the study.

Appendix 2
Test Item Characterization

Chemtura Corporation
12 Spencer St
Naugatuck, CT 06770

Analytical Services
www.chemtura.com

Certificate of Purity

Customer: Support for Toxicology Studies

Test Substance Name: MLA3202; Amides, tallow, N,N-bis(2-hydroxypropyl)

Physical Appearance: Liquid

CAS No.: 1454803-04-3

Ref. or Lot Number: RC-1045

Date of Analysis: revised March 18, 2016 (original issue March 7, 2016)

Percent Composition	Monoisotopic Mass (daltons)	Formula	Structure/ Identity
33.1	397.4	$C_{24}H_{47}NO_3$	C18:1 (oleic) tallow amides, N,N-bis(2-hydroxypropyl)
22.9	371.3	$C_{22}H_{45}NO_3$	C16:0 (palmitic) tallow amides, N,N-bis(2-hydroxypropyl)
13.6	395.4	$C_{24}H_{45}NO_3$	C18:2 (linoleic) tallow amides, N,N-bis(2-hydroxypropyl)
11.0	399.4	$C_{24}H_{49}NO_3$	C18:0 (stearic) tallow amides, N,N-bis(2-hydroxypropyl)
6.0	369.3	$C_{22}H_{43}NO_3$	C16:1 (palmitoleic) tallow amides, N,N-bis(2-hydroxypropyl)
3.2	419.3	$C_{26}H_{45}NO_3$	C20:4 (eicosatetraenoic) tallow amides, N,N-bis (2-hydroxypropyl)
2.0	393.3	$C_{24}H_{43}NO_3$	C18:3 (linolenic) tallow amides, N,N-bis(2-hydroxypropyl)
1.5	282.3	$C_{18}H_{34}O_2$	C18:1 (oleic) acid
1.1	421.4	$C_{26}H_{47}NO_3$	C20:3 (eicosatrienoic) tallow amides, N,N-bis (2-hydroxypropyl)
5.6			Sum of residual components (< 1% each)
100.0			Total

Blake Lewis 3/7/16
Blake Lewis Date
Analytical REACh Scientist, Analytical Services

Colin Moore for ABN 3/7/16
Albert J. Nitowski Date
Sr. Technology Manager
Analytical and Lab Support Services

Appendix 3
Summary Tables

Table 1
Dose-range Finding Test: Cytotoxicity of MLA-3202 (3 Hour Treatment)

dose (µg/ml)	cell count after 24 hours of subculture (cells/ml x10^5)	cell count after 48 hours of subculture (cells/ml x10^5)	SG(1) (x10^5 cells/ml)	RSG(2) (%)
	without metabolic activation			
SC	7.8	4.7	18	100
9.8	7.2	5.1	18	100
20	6.6	5.2	17	94
39	2.7	5.0	7	37
78	0.1 (4)	0.3	0	0
156 (3)	0.3 (4)	0.1	0	0
	with metabolic activation			
SC	5.7	5.6	16	100
9.8	4.6	6.7	15	97
20	4.5	6.1	14	86
39	5.9	5.8	17	107
78	3.9	6.5	13	79
156 (3)	0.3 (4)	0.1	0	0

Note: all calculations were made without rounding off
SC = solvent control = dimethyl sulfoxide

(1) = suspension growth

(2) = relative suspension growth

(3) = the test item precipitated in the exposure medium

(4) = since less than 1.25 x 10^5 c/ml were present, no subculture was performed

SG = Suspension growth = [Cell count after 24 hour of subculture (Day 1) /1.6 x 10^5] x [Cell count after 48 hours of subculture (Day 2)/1.25 x 10^5]*

* Or appropriate cell concentration

RSG = [$SG_{(test)}/SG_{(control)}$] x 100

Table 2
Dose-range Finding Test: Cytotoxicity of MLA-3202 (24 Hour Treatment)

dose (µg/ml)	cell count after 24 hours of treatment (cells/ml x10^5)	cell count after 24 hours of subculture (cells/ml x10^5)	SG[1] (x10^5 cells/ml)	RSG[2] (%)
	<u>without metabolic activation</u>			
SC	10.2	4.5	29	100
9.8	8.8	5.4	30	104
20	4.4	3.8	11	36
39	0.2 [4]	0.3	0	0
78	0.1 [4]	0.1	0	0
156 [3]	0.1 [4]	0.1	0	0

Note: all calculations were made without rounding off
SC = solvent control = dimethyl sulfoxide

(1) = suspension growth

(2) = relative suspension growth

(3) = the test item precipitated in the exposure medium

(4) = since less than 1.25 x 10^5 c/ml were present, no subculture was performed

SG = Suspension growth = [Day 0 cell count/1.25 x 10^5]* x [Day 1 cell count/1.25 x 10^5]*

* Or appropriate cell concentration

RSG = [$SG_{(test)}/SG_{(control)}$] x 100

Table 3
Experiment 1: Cytotoxic and Mutagenic Response of MLA-3202 in the Mouse Lymphoma L5178Y Test System

dose	RSG	CE day2	RCE	RTG	mutation frequency per 10^6 survivors		
(µg/ml)	(%)	(%)	(%)	(%)	total	small	large
			without metabolic activation				
			3 hour treatment				
SC1	100	93	100	100	63	15	47
SC2		102			55	13	40
1.25	115	89	91	105	67	11	55
2.5	105	91	94	98	49	17	30
5	11	78	80	9	59	18	40
10	113	85	87	99	61	7	52
20	98	86	89	87	69	28	39
25	71	93	95	67	36	13	23
30	34	91	94	32	48	14	33
35	1	104	106	1	72	23	47
MMS	94	60	62	58	447	190	227
			with metabolic activation				
			3 hour treatment				
SC1	100	101	100	100	41	16	24
SC2		97			47	11	36
5	99	98	99	98	55	14	40
20	96	91	92	89	55	13	41
30	97	85	86	84	61	16	43
50	89	105	107	95	62	23	37
60	81	89	90	73	52	11	40
70	76	120	121	92	56	14	40
80	41	89	90	37	54	14	39
90 (1)	19	83	84	16	74	23	49
CP	29	13	14	4	1507	947	492

Note: all calculations were made without rounding off
RSG = Relative Suspension Growth; CE = Cloning Efficiency; RCE = Relative Cloning Efficiency; RTG = Relative Total Growth;
SC = Solvent control = DMSO; MMS = Methylmethanesulfonate; CP = Cyclophosphamide
(1) Test item precipitated in the exposure medium

Table 4
Experiment 2: Cytotoxic and Mutagenic Response of MLA-3202 in the Mouse Lymphoma L5178Y Test System

dose (µg/ml)	RSG (%)	CE day2 (%)	RCE (%)	RTG (%)	mutation frequency per 10^6 survivors total	(small	large)
			without metabolic activation				
			24 hour treatment				
SC1	100	85	100	100	59	(15	43)
SC2		76			59	(11	47)
2.5	98	91	114	111	44	(15	28)
5	87	78	97	85	57	(34	22)
10	77	86	107	82	44	(21	22)
15	56	98	122	68	51	(24	26)
20	24	90	112	27	57	(22	33)
22.5	15	80	100	15	63	(43	18)
MMS	80	35	44	35	852	(479	312)

Note: all calculations were made without rounding off

RSG = Relative Suspension Growth; CE = Cloning Efficiency; RCE = Relative Cloning Efficiency;
RTG = Relative Total Growth; SC = Solvent control = dimethyl sulfoxide; MMS = Methylmethanesulfonate

Appendix 4
Detailed Tables

Abbreviations used:

SC, Solvent Control (SMSOn)

MMS, Methylmethanesulfonate

CP, Cyclophosphamide

RSG, Relative Suspension Growth

CE, Cloning Efficiency

SG, Suspension Growth

Table 5
Experiment 1 (rejected): Cell Counts (Without Metabolic Activation)

	DAY 0	DAY 1
dose (µg/ml)	total amount of cells before treatment ($x 10^6$)	cell count ($x10^5$ c/ml)
SC1	8	4.0
SC2	8	4.9
5 (1)	8	4.3
10 (1)	8	4.0
20 (1)	8	2.9
30 (1)	8	0.8
35 (1)	8	0.1
40 (1)	8	0.2
50 (1)	8	0.1
55 (1)	8	0.1
60 (1)	8	0.1
65 (1)	8	0.0
70 (1)	8	0.0
MMS	8	3.6

(1) = not used for the mutation experiment

Table 6
Experiment 1 (rejected): Cell Counts (With Metabolic Activation)

	DAY 0	DAY 1
dose (µg/ml)	total amount of cells before treatment ($x 10^6$)	cell count ($x10^5$ c/ml)
SC1	8	4.0
SC2	8	3.5
20 (1)	8	3.4
40 (1)	8	3.1
80 (1)	8	1.5
90 (1)(2)	8	0.4
100 (1)(2)	8	0.1
110 (1)(2)	8	0.1
120 (1)(2)	8	0.1
130 (1)(2)	8	0.1
140 (1)(2)	8	0.1
150 (1)(2)	8	0.1
CP	8	2.3

(1) = not used for the mutation experiment

(2) = test item precipitated in the exposure medium

Table 7
Experiment 1A: Cell Counts and Subculture Data (Without Metabolic Activation)

	DAY 0	DAY 1		DAY 2		
dose (µg/ml)	total amount of cells before treatment ($x 10^6$)	cell count ($x10^5$ c/ml)	subculture $x 10^6$ total amount [1]	cell count ($x10^5$ c/ml)	RSG (%)	SG
SC1	8	5.6	4.0	5.8	100	16
SC2	8	5.4	4.0	5.8		16
0.63 [2]	8	5.9	4.0	6.2	115	
1.25	8	6.3	4.0	5.8	115	
2.5	8	5.8	4.0	5.8	105	
5	8	5.9	4.0	6.0	111	
10	8	6.1	4.0	5.9	113	
20	8	5.2	4.0	6.0	98	
25	8	3.6	4.0	6.3	71	
30	8	1.7	4.0	6.3	34	
35	8	0.2	(3)	0.8	1	
40 [2]	8	0.1	(3)	0.2	0	
45 [2]	8	0.1	(3)	0.1	0	
50 [2]	8	0.1	(3)	0.1	0	
MMS	8	5.1	4.0	5.9	94	

Note: all calculations were made without rounding off

[1] = cell density 1.25×10^5 c/ml

[2] = not used for the mutation experiment

[3] = since less than 1.25×10^5 c/ml were present, no subculture was performed

Table 8
Experiment 1A: Cell Counts and Subculture Data (With Metabolic Activation)

	DAY 0	DAY 1		DAY 2		
dose (µg/ml)	total amount of cells before treatment ($x 10^6$)	cell count ($x10^5$ c/ml)	subculture $x 10^6$ total amount [1]	cell count ($x10^5$ c/ml)	RSG (%)	SG
SC1	8	5.7	4.0	6.7	100	19
SC2	8	5.7	4.0	6.4		18
5	8	5.6	4.0	6.6	99	
10 [2]	8	5.3	4.0	6.5	92	
20	8	5.7	4.0	6.3	96	
30	8	5.4	4.0	6.7	97	
40 [2]	8	5.5	4.0	7.5	110	
50	8	5.2	4.0	6.4	89	
60	8	4.3	4.0	7.0	81	
70	8	4.2	4.0	6.8	76	
80	8	2.3	4.0	6.6	41	
90 [4]	8	1.2	(3)	6.0	19	
100 [2] [4]	8	0.1	(3)	0.4	0	
CP	8	2.7	4.0	4.0	29	

Note: all calculations were made without rounding off

[1] = cell density 1.25×10^5 c/ml

[2] = not used for the mutation experiment

[3] = since less than 1.25×10^5 c/ml were present, no subculture was performed

[4] = test item precipitated in the exposure medium

Table 9
Experiment 1: Selection Data and Cloning Efficiency

dose (µg/ml)	mutant colonies: number of wells with mutants per selection plate [1]										total number of mutants			cloning efficiency (at day 2): n°. of empty wells per cloning plate		total number of empty wells	CE x 100%	mutation frequency per 10^6 survivors		
	1		2		3		4		5					1	2			total	small	large
without metabolic activation																				
	s	l	s	l	s	l	s	l	s	l	s	l	s+l							
SC1	2	6	4	11	3	8	1	6	3	9	13	40	53	35	41	76	93	63	15	47
SC2	5	9	1	7	2	7	2	8	3	7	13	38	51	31	38	69	102	55	13	40
1.25	1	7	4	9	2	11	2	4	0	14	9	45	54	35	44	79	89	67	11	55
2.5	1	4	4	4	6	5	1	4	3	9	15	26	41	31	46	77	91	49	17	30
5	1	4	3	4	2	5	2	8	5	8	13	29	42	52	36	88	78	59	18	40
10	2	7	0	7	1	10	0	10	3	7	6	41	47	39	43	82	85	61	7	52
20	7	10	4	3	5	7	3	7	4	4	23	31	54	39	42	81	86	69	28	39
25	1	5	2	4	3	1	3	3	2	7	11	20	31	39	37	76	93	36	13	23
30	3	3	1	8	2	5	3	7	3	5	12	28	40	44	33	77	91	48	14	33
35	3	10	5	8	2	5	7	12	5	10	22	45	67	32	36	68	104	72	23	47
MMS	14	7	9	16	12	18	13	8	12	13	104	123	227	53	52	105	60	447	190	227
	11	14	9	14	12	7	6	17	6	9										
with metabolic activation																				
	s	l	s	l	s	l	s	l	s	l	s	l	s+l							
SC1	4	4	4	5	3	4	3	6	1	4	15	23	38	34	36	70	101	41	16	24
SC2	2	10	1	5	1	3	2	9	4	5	10	32	42	35	38	73	97	47	11	36
5	3	7	2	8	5	10	1	3	2	8	13	36	49	38	34	72	98	55	14	40
20	3	5	1	10	3	6	1	6	3	8	11	35	46	37	40	77	91	55	13	41
30	2	7	3	7	0	9	5	5	3	6	13	34	47	44	38	82	85	61	16	43
50	1	7	5	5	6	9	8	7	3	8	23	36	59	32	35	67	105	62	23	37
60	3	3	0	2	2	8	4	6	0	14	9	33	42	41	38	79	89	52	11	40
70	1	7	2	10	3	15	4	5	6	7	16	44	60	34	24	58	120	56	14	40
80	2	8	5	8	2	8	1	3	2	5	12	32	44	40	39	79	89	54	14	39
90 [2]	1	3	4	5	4	8	9	12	0	9	18	37	55	44	40	84	83	74	23	49
CP	11	9	10	4	10	4	7	5	15	1	114	61	175	78	90	168	13	1507	947	492
	13	8	7	8	13	7	15	9	13	6										

s = small colonies
l = large colonies
[1] = Solvent controls and treatment groups five plates with 2000 cells/well and the positive controls ten plates with 1000 cells/well
[2] = Test item precipitated in the exposure medium

Table 10
Experiment 2: Cell Counts and Subculture Data (Without Metabolic Activation)

	24 HOUR TREATMENT			DAY 1		DAY 2		
dose (µg/ml)	total amount of cells before treatment ($x 10^6$)	total amount of cells after treatment ($x 10^6$)	subculture $x 10^6$ total amount [1]	cell count ($x10^5$ c/ml)	subculture $x 10^6$ total amount [1]	cell count ($x10^5$ c/ml)	RSG (%)	SG
SC1	6	19.8	4.0	5.9	4.0	7.6	100	95
SC2	6	19.6	4.0	6.8	4.0	6.6		94
2.5	6	19.0	4.0	6.5	4.0	7.0	98	
5	6	18.0	4.0	6.2	4.0	6.9	87	
10	6	15.8	4.0	6.3	4.0	6.8	77	
15	6	12.8	4.0	5.9	4.0	6.5	56	
20	6	7.4	4.0	4.9	4.0	5.9	24	
22.5	6	5.0	4.0	5.0	4.0	5.4	15	
25 [2]	6	4.0	3.9	4.0	4.0	4.1	7	
27.5 [2]	6	2.2	2.2	2.9	4.0	4.8	4	
30 [2]	6	1.0	1.0	0.9	(3)	3.3	1	
35 [2]	6	0.8	0.8	0.4	(3)	1.5	<1	
MMS	6	17.6	4.0	6.7	4.0	6.0	80	

Note: all calculations were made without rounding off

[1] = cell density 1.25×10^5 c/ml

[2] = not used for the mutation experiment

[3] = since less than 1.25×10^5 c/ml were present, no subculture was performed

Table 11
Experiment 2: Selection Data and Cloning Efficiency

dose (µg/ml)	mutant colonies: number of wells with mutants per selection plate (1)										total number of mutants			cloning efficiency (at day 2): n°. of empty wells per cloning plate		total number of empty wells	CE x 100%	mutation frequency per 10^6 survivors		
	1		2		3		4		5					1	2			total	small	large
without metabolic activation																				
	s	l	s	l	s	l	s	l	s	l	s	l	s+l							
SC1	1	5	4	11	2	5	1	6	4	7	12	34	46	40	42	82	85	59	15	43
SC2	2	8	1	11	2	5	2	4	1	5	8	33	41	50	40	90	76	59	11	47
2.5	7	4	0	8	1	5	2	4	3	3	13	24	37	38	39	77	91	44	15	28
5	8	0	5	10	3	2	4	1	5	3	25	16	41	45	43	88	78	57	34	22
10	2	5	3	4	1	4	6	3	5	2	17	18	35	43	38	81	86	44	21	22
15	7	4	5	10	4	3	4	4	2	3	22	24	46	35	37	72	98	51	24	26
20	4	4	7	10	2	11	4	2	2	1	19	28	47	38	40	78	90	57	22	33
22.5	5	4	5	4	7	1	9	4	6	1	32	14	46	41	45	86	80	63	43	18
MMS	12 12	18 7	14 14	9 10	21 16	9 10	14 14	7 10	14 18	8 12	149	100	249	67	68	135	35	852	479	312

s = small colonies
l = large colonies
(1) = Solvent controls and treatment groups five plates with 2000 cells/well and the positive controls ten plates with 1000 cells/well

Appendix 5
Historical Control Data

Table 12
Historical Control Data of the Spontaneous Mutation Frequencies of the Solvent Controls for the Mouse Lymphoma Assay

	Mutation frequency per 10^6 survivors		
	- S9-mix		**+ S9-mix**
	3 hour treatment	**24 hour treatment**	**3 hour treatment**
Mean	86	81	87
SD	23	26	28
n	220	202	273
Upper control limit (95% control limits)	135	135	145
Lower control limit (95% control limits)	37	28	28

SD = Standard deviation
n = Number of observations

Distribution historical negative control data from experiments performed between January 2013 and November 2016.

Table 13
Historical Control Data of the Mutation Frequencies of the Positive Controls for the Mouse Lymphoma Assay

	Mutation frequency per 10^6 survivors		
	- S9-mix		**+ S9-mix**
	3 hour treatment	**24 hour treatment**	**3 hour treatment**
Mean	857	688	1710
SD	246	187	815
n	110	102	139
Upper control limit (95% control limits)	1425	1124	4214
Lower control limit (95% control limits)	289	253	-793

SD = Standard deviation
n = Number of observations

Distribution historical positive control data from experiments performed between January 2013 and November 2016.